Colony-forming Ability *in vitro* and Clonology of Colorectal Cancer

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One hundred and twenty-three specimens (83 primary and 40 metastatic) of colorectal cancers from 102 patients were subjected to the human tumor clonogenic assay (HTCA) to determine the clonogenicities of tumor specimens stratified by the degree of histological differentiation and of tumor progression. No statistically significant differences in the clonogenicity were observed among the tumors with different histological differentiation. Viable malignant cells were abundant in the primary tumor specimens with submucosal (sm) and muscular layer (pm) invasion, and decreased significantly in number per gram of wet specimen as the tumors progressed ($P < 0.05$). Clonogenicity was also higher in "sm" and "pm" primary tumor specimens, and decreased as the tumors advanced ($P < 0.05$). These findings suggest that, at the onset or in the relatively early phase of the tumor progression, tumors are medullary and have a large fraction of clonogenic cells, and that they then develop more stroma while losing the clonogenic cell fraction by tumor progression. HTCA appears to be still valuable for analysis of the changes in biological properties over time in large bowel tumors.

Key words: Human tumor clonogenic assay — Colony-forming ability — Tumor progression

The human tumor clonogenic assay (HTCA²) has improved significantly over the past 12 years since Hamburger and Salmon¹ first established it in 1977. It has been applied to various human tumors to predict the response of patients to chemotherapy.²⁻⁴ Many reports have documented the potential clinical value of the assay,⁵⁻⁷ but some have also pointed out limitations.⁸⁻¹⁰

Since 5-fluorouracil was introduced as the first effective agent against colorectal cancers, further advances in chemotherapy for colorectal cancer have been very slow. Each tumor has its own characteristic time-course history, histological grade, and biological properties. Determination of the biological properties of each tumor and the clinical application of such results may be necessary to improve the chemotherapy of colorectal cancer.

The aim of this study was to elucidate the biological characteristics of subpopulations of cancers of the large bowel by the use of HTCA.

MATERIALS AND METHODS

Tumor cell collection One hundred and twenty-three colorectal cancer specimens from 102 patients were studied by means of HTCA. They included 83 primary tumors and 35 locoregional lymph node specimens. The HTCA was performed according to the modified method of Hamburger and Salmon, as previously reported.^{11,12} Specimens obtained at surgery were cut vertically in half,

and one was used for histological determination and the other for the HTCA. The former was fixed with 10% formalin solution and hematoxylin-eosin stain was used for determination of the degree of histological differentiation and the depth of tumor invasion, according to the General Rules for Pathological Studies on Cancer of Colon, Rectum and Anus.¹³ The other half was placed in CEM medium (MA Bioproducts, Walkersville, Md.) supplemented with 1% penicillin, 1% streptomycin, and 15% heat-inactivated fetal calf serum (Flow Laboratories, Inc., McLean, Va.). Cell suspensions were prepared enzymatically as previously described.^{11,12} In brief, the specimens were minced with sterile scalpels and placed in 75 cm² trypsinizing flasks with prewarmed Hanks' balanced salt solution (Flow Laboratories) containing 0.03% DNase (500,000 units/ml) and 0.14% collagenase type I (all from Calbiochem-Behring Corp., La Jolla, Calif.). Ten to 20 ml of enzyme medium was used per gram of wet specimen. Suspensions were digested enzymatically for 60 to 90 min at 37°C, filtered through two layers of gauze, and then washed. After these procedures, aliquots of single cell suspension were prepared for determining the yield and viability of large nucleated cells by trypan blue dye exclusion. Papanicolaou stain was used for differentiation of malignant cells from contaminating non-malignant cells.

HTCA Cells were cultured as described previously.^{11,12} In brief, layers of 1 ml of 0.5% Bacto-agar (Difco, Lab. Detroit, Mich.) in supplemented CEM were prepared in 35 × 10 mm plastic Petri dishes (Linbro 76-247-05; Flow Laboratories). Cells to be tested were suspended in 1 ml of 0.3% agar in supplemented CEM and poured over these underlayers. The final concentration of large

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² Abbreviations: HTCA, human tumor clonogenic assay; CEM, Chee's modification of essential medium; sm, submucosal invasion; pm, muscular invasion; ss, subserosal invasion; s, serosal invasion; si, invasion to adjacent organs.

nucleated malignant cells in each culture was 5×10^5 viable cells/ml. The plates were placed in a humidified incubator at 37°C in the presence of 6% CO_2 . Cultures were examined with an inverted microscope at $\times 4$ and $\times 10$ magnification. Colonies were defined as clusters greater than $60 \mu\text{m}$ in diameter. The maximum number of colonies per dish was reached within 3 weeks, at which time colony counts were made with an automatic particle counter (CP-3000, Shiraimatsu Co., Osaka). All plating was done in triplicate and the colony counts were expressed as the average of the three plates. A 10% formalin solution (1 ml) was poured over the top layer of one plate and the preparation was stored at 4°C . This plate was counted on the day of colony counting to obtain the background colony counts. Experiments were considered evaluable for assessment of chemosensitivity if (a) the net number of newly formed colonies in the untreated controls was greater than 30; (b) the mercuric chloride ($100 \mu\text{g}/\text{ml}$)-treated dishes showed at least 70% inhibition of colony formation when compared to the controls; and (c) the coefficient of variation for the newly formed colony count of the control group was less than 50%.

Statistical method Differential comparisons between two groups of data were performed by using Student's *t* test.

RESULTS

Colony-forming ability of primary versus metastatic specimens In the assays considered evaluable, the number of colonies per control dish was 113 ± 168 (mean $\pm$ standard deviation (SD)) ($n=53$) for the primary tumor specimens, 74 ± 76 ($n=21$) for the metastatic specimens and 106 ± 157 ($n=74$) overall (Table I). Colony-forming efficiency thus ranged from 0.006 to 0.27% of viable malignant cells with a median efficiency of 0.01% (50 colonies per dish). The clonogenicity of the primary tumor specimens was significantly higher than that of the metastases ($P > 0.01$). A similar tendency was also recognized in 11 patients in whom assessment of the assays was made for both the primary tumor and metastatic specimens. The results were 95 ± 117 for the primaries and 55 ± 61 for the metastases, with the difference not being statistically significant ($0.1 > P > 0.05$) (Table II).

Colony-forming ability of subpopulations of the primary tumor specimens stratified by the degree of histological differentiation Colony counts of the subpopulations of the primary tumor specimens were 112 ± 184 ($n=33$) for the well-differentiated adenocarcinomas, 107 ± 105 ($n=13$) for the moderately differentiated tumors, 120 ± 73 ($n=4$) for the poorly differentiated tumors, and 164 ± 60 ($n=3$) for the other histological types (Fig. 1). No statistically significant differences were recognized be-

Table I. Colony-forming Ability of the Primary versus the Metastatic Specimens

Specimen	n	Colony count (mean $\pm$ SD)	
Primary	53	113 ± 168	] $P < 0.01$
Metastasis	21	74 ± 76	
Overall	74	106 ± 157	

Table II. Colony-forming Ability in the Patients ($n=11$) in whom Both the Primary and the Lymph Node Specimens were Evaluably Assayed

Specimen	Colony count (mean $\pm$ SD)	
Primary	95 ± 117	] $0.1 > P > 0.05$
Lymph node metastasis	55 ± 61

Fig. 1. Colony-forming ability of the primary tumor specimens stratified by the histological differentiation.

tween the well, moderately, and poorly differentiated adenocarcinomas.

The percentage of assays evaluable was 59% for well-differentiated adenocarcinomas, 57% for moderately differentiated tumors, and 57% for poorly differentiated tumors (Table III). Overall, the evaluable percentage was 60% (74/123). These percentages generally corresponded to the clonogenicities of the specimens.

Viable malignant cells of wet primary specimens stratified by the degree of the primary tumor progression The number of viable malignant cells (range 1–100% with a median viability of 70%) per gram of the wet primary specimens was $1.20 \times 10^7 \pm 1.20 \times 10^7$ ($1.05 \times$

Table III. Evaluable Percentages of the Specimens Stratified by the Histological Differentiation

Specimen	Histological differentiation					Total
	well	moderately	poorly	mucinous	miscellaneous	
Primary	33/54 (61%)	13/19 (68%)	4/7 (57%)	1/1 (100%)	2/2 (100%)	53/83 (64%)
Metastasis	13/24 (54%)	4/11 (36%)	—	3/4 (75%)	1/1 (100%)	21/40 (53%)
Overall	46/78 (59%)	17/30 (57%)	4/7 (57%)	4/5 (80%)	3/3 (100%)	74/123 (60%)

Evaluable/assayed specimens.

Fig. 2. Viable malignant cells per gram of wet primary specimens.

Fig. 3. Colony-forming ability of the primary specimens.

10^6 – 3.11×10^7 , $n=4$) for tumors with submucosal (sm) invasion, $7.92 \times 10^6 \pm 6.12 \times 10^6$ (1.90×10^6 – 2.00×10^7 , $n=8$) for tumors invading the muscular layer (pm), $6.10 \times 10^6 \pm 5.54 \times 10^6$ (5.0×10^5 – 2.26×10^7 , $n=21$) for subserosal invasion (ss), $4.05 \times 10^6 \pm 3.01 \times 10^6$ (4.0×10^5 – 1.16×10^7 , $n=14$) for serosal invasion (s), and $3.10 \times 10^6 \pm 2.88 \times 10^6$ (3.0×10^5 – 8.33×10^6 , $n=6$) for tumors invading adjacent organs (si) (Fig. 2). The numbers were significantly higher in “sm” and “pm” tumors ($P<0.05$).

Colony-forming ability of subpopulations of the primary tumor specimens stratified by the degree of primary tumor progression The colony counts of the subpopulations of the primary tumor specimens were 218 ± 125 (30–331, $n=3$) for “sm” tumors, 124 ± 81 (44–310, $n=8$) for “pm” tumors, 80 ± 91 (33–449, $n=21$) for “ss” tumors, 93 ± 96 (35–409, $n=14$) for “s” tumors, and 63 ± 56 (30–183, $n=6$) for “si” tumors (Fig. 3). Clono-

Table IV. Evaluable Percentages of the Specimens Stratified by the Primary Tumor Progression

Specimen	Tumor progression					Total
	sm	pm	ss	s	si	
Primary	4/4 (100%)	8/8 (100%)	21/36 (61%)	14/22 (64%)	6/13 (46%)	53/83 (64%)
Metastasis	0/2 (0%)	2/2 (100%)	4/10 (40%)	8/13 (62%)	7/13 (54%)	21/40 (53%)
Overall	4/6 (67%)	10/10 (100%)	25/46 (54%)	22/35 (63%)	13/26 (50%)	74/123 (60%)

Evaluable/assayed specimens.

genicity was higher in "sm" and "pm" tumor specimens than in specimens from more invasive tumors ($P < 0.05$).

The percentage of evaluable assays is shown in Table IV. The percentage generally corresponded to the clonogenicity of the specimen.

DISCUSSION

The HTCA has been evaluated in various human malignant diseases, with some critical papers having been published recently. Many factors, such as tumor cell heterogeneity, *in vivo* drug delivery, and multi-log cell killing ability, limit the value of the HTCA as a predictor of clinical response. To achieve an improvement in chemotherapy, it is necessary to apply regimens specifically according to the biological properties of each tumor. The clonogenic or colony-forming cell fraction of the tumor is considered to be one of the main factors affecting tumor growth, although G_0 -phase stem cells cannot be evaluated in the HTCA. No difference of colony-forming ability across the histological spectrum of colorectal cancer was found in this study (Fig. 1). The number of viable malignant cells per gram of the wet primary specimens was higher in "sm" and "pm" tumors and a trend for a decrease to occur as the primary tumors advanced was observed (Fig. 2). The ratio of colony-forming cells to viable cells per gram of wet specimen represented the control colony count per dish (namely, clonogenicity). In other words, the colony count per dish represented the clonogenic or colony-forming cell fraction of the tumor cells in this assay. Colony-forming ability was higher in the "sm" and "pm" primary tumor specimens, and a tendency for it to decline as the tumor advanced was observed (Fig. 3). These findings suggest that, at the onset or in the relatively early phase of the tumor progression, tumors will be medullary (cancer cell-rich) and have a large fraction of clonogenic cells. As the tumors progress, they then develop more stroma and the clonogenic cell fraction is lost. This hypothesis might also be supported by the fact that the locoregional lymph node specimens had a good colony-forming ability in some cases where the primary tumor was advanced, had abundant stroma, and had a low clonogenicity. Colony counts were, for instance, 63 ± 56 ($n=6$) for the primary

versus 90 ± 81 ($n=7$) for the locoregional lymph node specimens of "si" tumors, although this difference was not statistically significant ($P > 0.05$).

Colony-forming cells in this assay were those which divided at least five times ($2^5 = 32 > 30$ cells) within about 3 weeks. Slowly growing tumor cells with a division time of more than 4 days, even with no cell loss, were not regarded as clonogenic cells in this assay (4 days $\times$ 5 divisions = 20 days $\approx$ 3 weeks). Such tumors may not be suitable for assessment by the HTCA. Cells which divided only a few times did not form a cluster containing more than 30 cells. The low fraction of clonogenic cells in the advanced primary tumor specimens may suggest that some fraction of the tumor cells stops cycling and enters the G_0 -phase after a number of cell divisions, so that the clonogenic fraction defined in this assay becomes smaller. Alternatively, the stromal development that becomes evident as tumors advance may reduce clonogenicity by prolongation of cell division time. In a rapid assay using ^3H -thymidine uptake,¹⁴⁻¹⁶ cells which cycle at least once are evaluated. The HTCA and this rapid ^3H -thymidine assay are probably only suitable to estimate the characteristics of tumor cells which are actively cycling.

In the study of the predictive value of HTCA, clinical responses have usually been evaluated in advanced lesions. Such tumors include many G_0 -phase cells together with the cells which cycle more than several times as well as clonogenic cells. In the HTCA, only the clonogenic cells are targeted and evaluated. The predictive value of the HTCA should be assessed not only in terms of changes of evaluable tumor size after drug treatment but also in terms of patient prognosis.

To achieve more effective cancer therapy, it is thought to be necessary to analyze the biological properties of tumors. The HTCA is still a useful method for the analysis of the changes in biological properties over time of malignant tumors.

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